TITLE OF THE INVENTION

Peptide Fragments of Cholera Toxin B or Enterotoxin B as Vaccine Adjuvants

RELATED APPLICATION DATA

This application claims priority of international application number PCT/GB99/02970, filed internationally 7 September 1999, and GB 9819484.8, filed 7 September 1998.

FIELD OF THE INVENTION

The present invention relates to a substance.

In particular, the present invention relates to a substance that is capable of displaying one or more properties that are useful in medicine.

By way of example, the substance is useful for use as an immunomodulator and/or an adjuvant and/or an inhibitor of toxin-induced diarrhoea.

More in particular, the present invention relates to the use of an immunomodulatory substance in modulating an immune response - such as that associated with an autoimmune disease.

More in particular, the present invention relates to the use of the substance as an adjuvant when given in combination with a related or an unrelated antigen.

More in particular, the present invention relates to the use of the substance for inhibiting toxin-induced diarrhoea.

The present invention also relates to an assay for screening for agents capable of interacting with the substance of the present invention.

BACKGROUND OF THE INVENTION

Escherichia coli (E. coli) heat labile enterotoxin (Etx) and its closely related homologue, cholera toxin (Ctx) from *Vibrio cholerae,* are examples of protein toxins which bind to glycolipid receptors on host cell surfaces. Each toxin consists of six noncovalently linked polypeptide chains, including a single A subunit (27 kDa) and five identical B subunits (11.6 kDa) which bind to GM-1 ganglioside receptors found

on the surfaces of mammalian cells (Nashar *et al* 1996 Proc Natl Acad Sci 93: 226-230). The A subunit is responsible for toxicity possessing adenosine diphosphate (ADP) ADP-ribosyltransferase activity, whereas the B subunits (EtxB and CtxB) are non-toxic oligomers which bind and cross-link a ubiquitous cell surface glycolipid ganglioside, called GM-1, thus facilitating A subunit entry into the cell.

In contrast to the poor immunogenicity of the A subunit alone, both EtxB and CtxB are exceptionally potent immunogens and their respective holotoxins, Etx and Ctx (which comprise the A and B subunits) are known to be potent adjuvants when given orally in combination with unrelated antigens (Ruedl *et al* 1996 Vaccine 14: 792-798; Nashar *et al* 1993 Vaccine 11: 235; Nashar and Hirst 1995 Vaccine 13: 803; Elson and Ealding 1984 J Immunol 133: 2892; Lycke and Holmgren 1986 Immunology 59: 301). Because of their immunogenicity, both EtxB and CtxB have been used as carriers for other epitopes and antigens (Nashar *et al* 1993 *ibid*) and have been used as components of vaccines against cholera and *E.coli* mediated diarrhoeal diseases (Jetborn *et al* 1992 Vaccine 10: 130).

Several studies have been carried out on the immunodominant epitope of the CtxB and EtxB subunits with a view to developing a vaccine against the cholera toxin and heat labile *E.coli* toxin. By way of example, the following disclosures represent some of the work that has been carried out in this area.

UK Patent Application No. 2 415 419A discloses a synthetic vaccine against cholera and against heat labile toxin of *E. coli* comprising a conjugate of a carrier with a synthetic polypeptide corresponding to part of the sequence of CtxB.

WO 85/02611 discloses synthetic polypeptides corresponding to particular sequences of EtxB which are deemed useful as conjugates or as an active ingredient to raise antibodies against the B subunit and for protecting a host animal against infection by enterotoxins.

WO 89/10967 discloses an amino acid sequence which represents residue numbers 50-64 of the CtxB which can be used in combination with an epitopes of a heterologous organism, such as *Flagellum* and/or *Salmonella,* in vaccine formulations with a view to providing protection against infection by the heterologous organism or to providing protection against conditions or disorders caused by an antigen of the organism.

WO 90/03437 relates to a hybrid protein which fuses the CtxB subunit with the active sequence of a heterologous antigen which is deemed useful for vaccination purposes, particularly to help the stabilisation of heterologous antigens in the intestinal environment.

WO 94/06465 relates to amino acid fragments which are linked, either with or without a linker, to an appropriate carrier such that the amino acid fragment linked to the carrier generates an opsonic or protective immune response to the epitopes of the fragment.

WO95/29701 discloses a vaccine against *Vibrio cholera* which comprises a conjugate of cholera toxin B subunit (CTB) or a synthetic fragment peptide which consists of a portion thereof, such as peptide CTP 3 comprising the 50-64 amino acid sequence of the B chain linked to an inert carrier.

WO96/26282 discloses an expression systems for expressing gene products from recombinant *Bordetella* strains wherein the gene product may be a cholera toxin molecule.

WO96/34893 discloses hybrid molecules between EtxB and CtxB which may be useful as a vaccine and to prevent and/or treat enterotoxin-induced illness in an individual.

WO 98/21344 discloses an EtxB subunit which is modified to include an inserted antigenic peptide. The chimeric antigen-EtxB molecule is used to elicit an antibody response against an antigenic peptide in host animals.

These studies related to either the use of (i) a peptide comprising part of the sequence of CtxB/EtxB or (ii) a peptide comprising part of the sequence of CtxB/EtxB coupled to a second entity (such as an antigen) to induce and/or maximise the immunological response to that peptide. Accordingly, these documents relate to the use of an immunodominant epitope of CtxB/EtxB or parts thereof as an immunogen in inducing an immunological response against these subunits with a view to developing immunity against cholera and/or *E.coli* mediated diarrhoea diseases. WO 91/07979 discloses a chimeric protein which includes a portion of CtxB and an epitope region of a desired antigen which are designed for use as a vaccine to elicit an immune response in a subject to a desired antigen. In this regard, the portion of CtxB is being used as an adjuvant but not as an immunomodulator. Accordingly, none of the above cited documents relates to the use of a CtxB/EtxB peptide or part thereof as an immunotherapeutic capable of modulating the immune response.

We have shown that the EtxB subunit is capable of acting as an immunomodulator in immune disorders. By way of example, we have disclosed in WO 97/02045 that EtxB binds to GM-1 ganglioside receptors which are found on the surfaces of mammalian cells and that this binding induces differential effects on lymphocyte populations including a specific depletion of CD8+ T cells and an associated activation of B cells. These effects are absent when a mutant EtxB protein (G33D) (lacking GM-1 binding activity) is employed. Consequently, these experimental results would suggest that all of the functionalities associated with EtxB and CtxB are attributable to the capacity of the EtxB and CtxB subunits to bind to the GM-1 receptor since mutants lacking the capacity to bind GM-1 (such as EtxB (G33D)) fail to act as adjuvants or immunomodulators. Thus, the prior art to date has suggested that immunomodulation and other effects of Etx and Ctx are mediated through GM-1 binding. However, until now, no investigations have been carried out on CtxB/EtxB mutants which retain the capacity to bind to the GM-1 receptor but which lack an immunomodulatory effect.

We have suprisingly found that not all of the effects of Etx and Ctx are mediated through GM-1 binding.

SUMMARY ASPECTS OF THE INVENTION

In accordance with the present invention we have now found that the immunomodulation and some other effects of Etx and Ctx are not mediated through GM-1 binding.

Aspects of the present invention are presented in the accompanying claims and in the following description and discussion.

In one aspect of the present invention there is provided a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB; but wherein the substance is not capable of exhibiting GM-1 binding activity.

In a preferred aspect of the present invention there is provided a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to loop of EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

In a highly preferred aspect of the present invention there is provided a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to the β4-α2 loop of EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

The substance of the present invention can be an amino acid sequence or a chemical derivative thereof. The substance may be a synthetic peptide or a synthetic peptide variation - such as a retroinverso D peptide. The substance may even be an organic compound or other chemical. The latter examples are example of mimetics of SEQ ID No. 2.

~~The susbstance of the present invention is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB.~~

The term "same as or is similar to" is a qualitative term rather than a quantitative term. In this respect, it may be desirable to have an increased binding affinity.

An assay for determining whether a substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB would be readily determinable to those skilled in the art. For example, the assay may measure and/or determine an effect on cell populations, such as lymphocyte cell populations. These effects can include but are not limited to an induction of apotosis in CD8+ T cells , the enhanced activation of CD4+ T cells and the polyclonal activation of B cells. In addition, or in the alternative, the assay could be based on determining and/or measuring particular cell surface marker(s) indicative of activation of certain intracellular events (e.g. meauring an increase in CD25 expression).

The susbstance of the present invention is not capable of exhibiting GM-1 binding activity.

An assay for determining the lack of GM-1 binding activity would be readily determinable to those skilled in the art. For example, the assay may utilise GM-1 bound to a solid support and wherein the substance is then passed across the bound GM-1. Elution of the substance is indicative that it does not bind to GM-1. In a more preferred aspect, the assay is that described in WO 97/02045.

It is to be noted that the binding activity of the substance is not necessarily dependent on a primary binding event as is found with full length Ctx and EtxB subunits. With full length Ctx and EtxB, the primary binding activity is GM-1 binding activity. In this regard, the substance may exhibit a single binding event. However, for some cases, the substance may possess the capability of having more than one binding activity.

Preferably, the substance is substantially isolated and/or substantially pure.

As used herein, the terms "isolated" and "purified" refer to molecules, either nucleic or amino acid sequences, that are removed from their natural environment and/or isolated or separated from at least one other component with which they are naturally associated. A protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the substance and still be regarded as substantially isolated.

The present invention is based on the suprising finding that there are mutants which are capable of binding to the GM-1 receptor but which lack an immunomodulatory effect. These mutants facilitate the elucidatation of the mechanism by which the B subunits of Ctx and/or Etx act, particularly *vis-a-vis* an immunomodulatory effect.
Other aspects of the present invention are as follows.

A substance according to the present invention for use in medicine.

A substance according to the present invention for use as an immunomodulator.

A substance according to the present invention for use as an adjuvant.

A substance according to the present invention for use as an inhibitor of toxin-induced diarrhoea.

A substance according to the present invention wherein the substance additionally comprises an antigen or an antigenic determinant.

A pharmaceutical composition comprising the substance according to the present invention, optionally admixed with one or more pharmaceutically acceptable carrier(s), diluent(s) or excipient(s).

Use of a substance according to the present invention for use in the manufacture of a medicament that is capable of treating and/or preventing and/or modulating a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder.

An assay method for determing one or agents that are capable of interacting with and/or affecting the substance according to the present invention; wherein the assay comprises contacting the substance with an agent to be tested, and then determining whether or not the agent affects the substance.

An agent identified by the assay method according to the present invention.

A method of treatment, comprising administering to a subject in need of treatment of and/or prevention of and/or modulation of a disease and/or condition associated with an immune disorder and/or a toxin mediated disorder a substance according to the present invention.

These aspects are presented under separate section headings. However, it is to be understood that the teachings under each section heading are not necessarily limited to that particular section heading.

IMMUNOMODULATOR

As used herein, the term "immunomodulator" means a substance that is capable of modulating the immune response by inducing, for example, a differential effect on cells, such as lymphocyte cells - preferably leading to induction of apoptosis in CD8+ T cells and/or enhanced activation of CD4+ cells and/or the polyclonal activation fo B cells.

The term "differential effect on leukocyte cells" may include but is not limited to a specific depletion of CD8+ cells (through for example apotosis), the enhanced activation of CD4+ T cells and/or an associated activation of B cells.

Preferably the immunomodulator is capable of downregulating the pathological response of Th1 and/or Th2-associated immune responses.

Preferably the immunomodulator is capable of upregulating the production of antibodies at mucosal surfaces.

ADJUVANT

As used herein, an "adjuvant" is a substance which non-specifically enhances the immune response to an antigen.

It also includes any substance which is capable of affecting the extent of the immune response to an entity such as an antigen and/or an antigenic determinant, by altering the antigenicity of the antigen or by altering the specific reactivity or the nonspecific effector associated mechanisms of the host such that an immune response is induced in a host cell and/or is guided in a particular direction. In one preferred aspect, the adjuvant is capable of acting as a mucosal adjuvant.

Preferably the adjuvant is capable of prolonging antigen presentation and providing a sustained immunologic memory in a mammalian subject.

ANTIGEN

As used herein, an "antigen" means an entity which, when introduced into an immunocompetent host, stimulates the production of a specific antibody or antibodies that can combine with the entity. The antigen may be a pure substance, a mixture of substance or soluble or particulate material (including cells or cell fragments). In this sense, the term includes any suitable antigenic determinant, auto-antigen, self-antigen, cross reacting antigen, alloantigen, xenoantigen, tolerogen, allergen, hapten, and immunogen, or parts thereof, as well as any combination thereof, and these terms are used interchangeably throughout the text.

An "allergen" includes any antigen that stimulates an allergic reaction, inducing a Type I hypersensitivity reaction.

Examples of common allergen sources include but are not limited to ragweed, rye, couch, wild oat, timothy, Bermuda, Kentucky blue, mugwortalder, birch, hazel, beech, Cupressae, oak, olive, *Aspergillus* spp., *Cladosporium* spp., *Alternaria* spp., Basidospores, Ascomyceteswheat, rye, oatcat, dog, horse, rabbit, guinea pig, hamsterbudgerigar, parrot, pigeon, duck, chicken, Dermatophagoides pteronyssinus, D.farinae, Euroglyphus maynei, cockroach, fly, locust, midge, seafood, legumes, peanuts, nuts, cereals, dairy products, eggs, fruits, tomatoes, mushrooms, alcoholic beverages, coffee, chocolate, penicillins, sulphonamides and other antibiotics, sulphasalazine, carbamazepine, bee and wasp stings, ant and mosquito bitesblood products, sera, vaccines, contrast media, drugs (including anti-asthma drugs and antibiotics).

ANTIGENIC DETERMINANT

The term "antigenic determinant" as used herein refers to a site on an antigen which is recognised by an antibody or T-cell receptor. Preferably it is a short peptide derived from or as part of a protein antigen. However the term is also intended to include glycopeptides and carbohydrate epitopes. The term also includes modified sequences

of amino acids or carbohydrates which stimulate responses which recognise the whole organism.

It is advantageous if the antigenic determinant is an antigenic determinant of the infectious agent (such as a bacterium or virus) which causes the infectious disease.

By way of example, if the infectious agent is EBV, the antigenic determinant may be an antigenic determinant of gp340 or gp350 or of a latent protein (for example EBNAs 1,2 3A, 3B, 3C and -LP, LMP-1, -2A and 2B or an EBER). If the infectious agent is an influenza virus, the antigenic determinant may be an antigenic determinant of a viral coat protein (for example haemagglutinin and neuraminidase) or of an internal protein (for example, nucleoprotein). If the infectious agent is selected from the group consisting of enteropathogenic, enterotoxigenic, enteroinvasive, enterohaemorrhagic and enteroaggregative *E.coli*, then the antigenic determinant may be an antigenic determinant of a bacterial toxin or adhesion factor.

It is also advantageous if the antigenic determinant is an antigenic determinant from an autoantigen.

AGENT

The agent can be an amino acid sequence or a chemical derivative thereof. The substance may even be an organic compound or other chemical. The agent may be a nucleotide sequences - which may be sense or anti-sense sequences. The agent may be an antibody. In one preferred aspect, the agent is a cell receptor that is engageable by the substance.

INHIBITOR OF TOXIN-INDUCED DIARRHOEA

The term "inhibitor of toxin-induced diarrhoea" includes any substance which is capable of affecting the activity of Etx/Ctx holotoxins such that the pathological consequences of Etx/Ctx, such as diarrhoea, may be avoided.

DETAILED DESCRIPTION OF THE INVENTION

The present invention demonstrates the highly surprising finding that:

(i) the substance of the present invention is capable of acting as an immunomodulator and/or an adjuvant and/or an inhibitor of toxin-induced diarrhoea which is capable of affecting enterotoxin mediated diarrhoeal diseases.

(ii) the substance of the present invention is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB. The activity of the substance of the present invention may be mediated by the "so-called" ß4-α2 loop of EtxB and CtxB, which is a flexible loop included within amino acid residues 45-65.

(iii) EtxB molecules with point mutations at three separate sites within the ß4-α2 loop (positions 51, 56 and 57) retain GM-1 binding activity, but lack other activities, such as toxicity and the capacity to upregulate CD25 and trigger apoptosis of CD8-positive T-cells. In addition, Ctx holotoxins comprising B subunits with mutations also show a defect in an ability to trigger electrogenic chloride secretion, the primary secretory event responsible for mediating diarrhorea. These finding are particularly surprising, since flexible loops are usually thought to serve only to join two elements of secondary structure together, and rarely have an important function themselves.

(iv) the binding activity of the substance is not necessarily dependent on a primary binding event as is found with full length Ctx and EtxB. With full length Ctx and EtxB, the primary binding activity is GM-1 binding activity.

Ctx/Etx TOXINS

As used herein, the term "Ctx" refers to the cholera toxin and the term "CtxB" refers to the B subunit of the cholera toxin. In other texts, these may sometimes be identified as CT or Ct or CTB or CtB respectively.

As used herein, the term "Etx" herein means the *E. coli* heat labile enterotoxin and the term "EtxB" is the B subunit of Etx. In other texts, these may sometimes be identified as LT or Lt and LTB or LtB respectively.

ß4-α2 LOOP

In one aspect, the present invention relates to an substance comprising the sequence EVPGSQH (SEQ ID No 2) which is capable of acting in a manner that is the same or is similiar to EtxB and/or CtxB or a variant thereof, or a homologue thereof, or a fragment thereof or a derivative thereof or a mimetic thereof but which is not capable of exhibiting GM-1 binding activity.

Without wishing to be bound by theory, we believe that the binding of the five Etx/Ctx B subunits to GM-1 is a high affinity interaction, which allows a relatively low affinity secondary binding activity of EtxB/CtxB to occur. This binding is mediated by the the ß4-α2 loop of EtxB/CtxB. The structure of the ß4-α2 loop of EtxB/CtxB can be understood by reference to the molecular structure of Etx as described in detail in Sixma *et al.* J. Mol. Biol. (1993) 230; 890-918) and as illustrated in Figure 1.

In summary, each B subunit of Etx or Ctx consists of a small N-terminal helix (α1), two three-stranded anti-parallel sheets (sheet I, composed of strands ß2, ß3, ß4 and sheet II, composed of strands ß1, ß5 and ß6), and a long α-helix (α2). The two ß sheets form a ß barrel. The loops joining these elements of secondary structure in the B subunit can be divided into two classes, referring to the two ends of the sheets. On one end of the subunit, the "narrow" (or "A") end, the loops are generally short,

involving the connections ß1-ß2, ß3-ß4, and α2-ß5 as well as the C-terminus. The subunit widens at the other end, with much longer loops connecting secondary structure elements α1-ß1, ß2-ß3, ß4-α2. The longest loop connects ß4 and α2 (hereinafter the "ß4-α2 loop"), includes the residues Glu 51 to Asp 59, and extends below the plane of the ß sheets. This loop is quite flexible, but according to Sixma *et al* (Nature (1992) **355**; 561-564) becomes distinctly less mobile after lactose binding. The present invention demonstrates that the ß4-α2 loop of EtxB/CtxB is responsible for the secondary binding activity and so the use of this loop in isolation from the rest of the EtxB/CtxB molecule (for example as a peptide), may permit the secondary binding activity to occur in the absence of the first. Selective mutation of the ß4-α2 loop, or a peptide derived from this loop, may be exploited with a view to increasing the affinity of the secondary binding activity. By increasing the affinity of the secondary binding activity, the interaction with GM-1 may be further obviated.

As used herein, the term "ß4-α2 loop of EtxB/CtxB" is the entity which is responsible for the secondary binding activity of the B subunits of toxins such as the cholera toxin and heat labile *E.coli* toxin. When the ß4-α2 loop is used in isolation from the rest of the EtxB and/or CtxB molecule (for example as a peptide), the secondary binding activity may occur in the absence of the first and is herein after referred to as an activity or binding activity.

Preferably the substance of the present invention comprises an isolated ß4-α2 loop of EtxB/CtxB.

Preferably the substance comprises a mimetic of the isolated ß4-α2 loop of EtxB/CtxB.

Preferably the substance comprises a mimetic of the isolated ß4-α2 loop of EtxB/CtxB with a high affinity binding activity.

Preferably the substance comprise a peptide of from about 5 to about 40 amino acids.

Preferably the peptide has less than 25 amino acids.

If the peptide is a fusion protein, preferably the peptide has greater than 25 amino acids.

Preferably the substance comprises the sequence VEVPGSQHIDSQ (SEQ ID No 3).

Preferably the substance comprises the sequence GATFQVEVPGSQHIDSQKKAI (SEQ ID No 4).

Preferably the substance comprises the sequence GETFQVEVPGSQHIDSQKKAI (SEQ ID No 5) derivable from residues 45-65 of porcine *E.coli.*

Preferably the substance comprises residues 45-65 derivable from EtxB of the human variant of *E.coli* derivable from EtxB of the porcine variant of *E.coli.*

AMINO ACID SEQUENCE

The present invention provides a substance comprising the amino acid sequences of the present invention which is capable of acting as an immunomodulator and/or an adjuvant and/or an an inhibitor of toxin-induced diarrhoea which is capable of affecting enterotoxin mediated diarrhoeal diseases. The substance may also be used in assays for the identification of one or more agents capable of interacting with and/or affecting the substance activity.

As used herein, the term "amino acid sequence" refers to peptide, polypeptide sequences, protein sequences or portions thereof.

AFFECT

The term "affect" includes modulation, such as treatment, prevention, suppression, alleviation, restoration, elevation, modification of the substance activity.

The term "modification" includes but is not limited to disabling, silencing, mutating, removing, enhancing, increasing, agonising, antagonising, decreasing or blocking the substance activity.

VARIANTS/HOMOLOGUES/DERIVATIVES

Preferred amino acid sequences of the invention are SEQ ID No 2 or SEQ ID No 3 or SEQ ID No 4 or SEQ ID No 5 or sequences obtainable from the substance of the present invention but also include homologous sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof.

Thus, the present invention covers variants, homologues or derivatives of the amino acid sequences presented herein, as well as variants, homologues or derivatives of the nucleotide sequence coding for those amino acid sequences.

In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least the 7 amino acids of SEQ ID No 2, for example as shown in the sequence listing herein. In particular, homology should typically be considered with respect to those regions of the sequence (such as amino acids at positions 51, 56 and 57) known to be essential for an activity which is the same or is similiar to EtxB and/or CtxB rather than non-essential neighbouring sequences. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention presented as SEQ ID No 2, SEQ ID No 3, SEQ ID No 4 and SEQ ID No 5 includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant entity retains an activity, preferably having at least the same and/or similiar activity as CtxB and/or EtxB.

SEQ ID No 2 or SEQ ID No 3 or SEQ ID No 4 or SEQ ID No 5 may be modified for use in the present invention. Typically, modifications are made that maintain the activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the activity.

The substance of the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

In one aspect, the present invention provides nucleotide sequences encoding the substance of the present invention capable of acting as a template or as targets in assays (such as a yeast two hybrid assay) for the identification of one or more agents and/or derivatives thereof capable of affecting the substance.

As used herein, the term "nucleotide sequence" refers to nucleotide sequences, oligonucleotide sequences, polynucleotide sequences and variants, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be DNA or RNA of genomic or synthetic or recombinant origin which may be double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof. Preferably, the term nucleotide sequence is prepared by use of recombinant DNA techniques (e.g. recombinant DNA).

Preferably, the term "nucleotide sequence" means DNA.

The substance encoding nucleotide sequence may be the same as the naturally occuring form for this aspect. Preferably the nucleotide sequence encoding the substance is a non-native nucleotide sequence - or is a variant, homologue, fragment or derivative thereof. Thus, in a preferred embodiment, the present invention does not cover the native nucleotide coding sequence according to the present invention in its natural environment when it is under the control of its native promoter which is also in its natural environment. For ease of reference, we have called this preferred embodiment the "non-native nucleotide sequence".

As used herein "naturally occurring" refers to an substance with an amino acid sequence found in nature.

As used herein "biologically active" refers to an substance having regulatory or biochemical functions of the naturally occurring substance.

VARIANTS/HOMOLOGUES/DERIVATIVES

VARIANTS/HOMOLOGUES/DERIVATIVES

The terms "variant", "homologue" or "derivative" in relation to the nucleotide sequence SEQ ID No 1 of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for an substance having an activity, preferably having at least the same activity as the SEQ SEQ ID No 2, SEQ ID No 3, SEQ ID No 4 and SEQ ID No 5 presented in the sequence listings.

As indicated above, with respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listing herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50 nucleotides in length.

As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring substance.

HYBRIDISATION

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

Nucleotide sequences of the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 75%, preferably at least 85 or 90% and more preferably at least 95% or 98% homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Preferred nucleotide sequences of the invention will comprise regions homologous to nucleotides comprising SEQ ID No 1 preferably at least 80 or 90% and more preferably at least 95% homologous to SEQ ID No 1.

The term "selectively hybridizable" means that the nucleotide sequence used as a probe is used under conditions where a target nucleotide sequence of the invention is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other nucleotide sequences present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P.

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related nucleotide sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3 Citrate pH 7.0). Where the nucleotide sequence of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the nucleotide sequence is single-stranded, it is to be understood that the complementary sequence of that nucleotide sequence is also included within the scope of the present invention.

EXPRESSION VECTORS

The nucleotide sequences of the present invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence in and/or from a compatible host cell. Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

FUSION PROTEINS

The substance of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the substance comprising the amino acid sequence of the present invention.

In one embodiment of the present invention, the fusion protein comprises an antigen or an antigenic determinant fused to the substance of the present invention. In this embodiment, the fusion protein is a non-naturally occuring fusion protein comprising a substance which may act as an adjuvant in the sense of providing a generalised stimulation of the immune system. The antigen or antigenic determinant may be attached to either the amino or carboxy terminus of the substance.

In another embodiment of the invention, the substance of the invention may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognized by a commercially available antibody.

In yet another embodiment, an assay for identifying an agent, such as a target receptor, or for an agent capable of regulating CD25 transcriptional activity may be conducted using a bound fusion protein.

ANTIBODIES

In yet another embodiment, an assay for identifying an agent, such as a target receptor, or for an agent capable of regulating CD25 transcriptional activity may be conducted using a bound fusion protein.

ANTIBODIES

In one embodiment of the present invention, the substance of the present invention may be an antibody. This antibody may be capable of acting as a mimetic of the present invention.

Antibodies may be produced by standard techniques, such as by immunisation with the substance of the invention or by using a phage display library.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody. Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in substance-A-239400. Neutralizing antibodies, i.e., those which inhibit biological activity of the substance polypeptides, are especially preferred for diagnostics and therapeutics.

In one embodiment, the invention also provides monoclonal or polyclonal antibodies to substances of the invention such as polypeptides or fragments thereof. Thus, the present invention further provides a process for the production of monoclonal or polyclonal antibodies to substances, such as polypeptides of the invention.

POLYCLONAL ANTIBODIES

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing a epitope(s) obtainable from an identifed agent and/or substance of the present invention. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are potentially useful human adjuvants which may be employed if purified the substance polypeptide is administered to immunologically compromised individuals for the purpose of stimulating systemic defence.

Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an epitope obtainable from an identifed agent and/or substance of the present invention contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

MONOCLONAL ANTIBODIES

Monoclonal antibodies directed against epitopes obtainable from an identifed agent and/or substance of the present invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of

monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

Monoclonal antibodies to the substance and/or identified agent of the present invention may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 Nature 256:495-497), the human B-cell hybridoma technique (Kosbor *et al* (1983) Immunol Today 4:72; Cote *et al* (1983) Proc Natl Acad Sci 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al* (1984) Proc Natl Acad Sci 81:6851-6855; Neuberger *et al* (1984) Nature 312:604-608; Takeda *et al* (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.

Antibodies, both monoclonal and polyclonal, which are directed against epitopes obtainable from an identifed agent and/or substance of the present invention are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the substance and/or agent against which protection is desired. Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful in therapy.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, Proc Natl Acad Sci 86: 3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

Antibody fragments which contain specific binding sites for the substance may also be generated. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al* (1989) Science 256:1275-128 1).

ASSAYS FOR IMMUNOMODULATORY SUBSTANCES

The immunomodulation of the immune response may be measured by transcriptional profiling, for example, by assaying for activation of transcription of CD25 cell surface marker by measuring the signal from a linked reporter gene.

REPORTERS

Preferably a wide variety of reporters may be used in the assay methods of the present invention with preferred reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a reporter gene may encode an enzyme which catalyses a reaction which alters light absorption properties.

Examples of reporter molecules include but are not limited to β-galactosidase, invertase, green fluorescent protein, luciferase, chloramphenicol, acetyltransferase, β-glucuronidase, exo-glucanase and glucoamylase. Alternatively, radiolabeled or fluorescent tag-labeled nucleotides can be incorporated into nascent transcripts which are then identified when bound to oligonucleotide probes.

In one preferred embodiment, the production of the reporter molecule is measured by the enzymatic activity of the reporter gene product, such as β-galactosidase.

ASSAYS FOR INHIBITORS OF TOXIN-INDUCED DIARRHOEA

The substance of the present invention or a derivative or homologue thereof and/or a cell line that expresses the substance of the present invention or a derivative or homologue thereof may be used to screen for agents (such as antibodies, peptides, organic or inorganic molecules) capable of affecting the activity of the substance. By way of example, any agent capable of inhibiting the activity of the substance may be screened for inhibitors of toxin-induced diarrhoea thereby identifying agents capable of affecting the cholera and/or enterotoxin mediated diarrhoeal diseases.

In one embodiment, the screens of the present invention may identify antagonists of the substance of the present invention, such as antibodies, peptides or small organic molecules which are capable of acting as inhibitors of toxin-induced diarrhoea

ASSAYS FOR AGENTS

Phage display may be employed in the identification of agents, such as a cell surface receptor that is engageable by the substance of the present invention. The positive identification of such a receptor may faciliate the use of combinatorial libraries to identify mimetics capable of acting in the same or a similiar manner as the substance of the present invention.

Phage display is a protocol of molecular screening which utilises recombinant bacteriophage. The technology involves transforming bacteriophage with a gene that encodes an appropriate ligand (in this case a candidate agent) capable of reacting with a target substance (or a derivative or homologue thereof) or the nucleotide sequence (or a derivative or homologue thereof) encoding same. The transformed bacteriophage (which preferably is tethered to a solid support) expresses the appropriate ligand (such as the candidate agent) and displays it on their phage coat. The entity or entities (such as cells) bearing the target substance molecules which recognises the candidate agent are isolated and amplified. The successful candidate agents are then characterised. Phage display has advantages over standard affinity

ligand screening technologies. The phage surface displays the candidate agent in a three dimensional configuration, more closely resembling its naturally occuring conformation. This allows for more specific and higher affinity binding for screening purposes.

ASSAYS FOR MIMETICS

In one embodiment, the screens of the present invention may identify mimetics of the substance of the present invention, such as antibodies, or other chemical compounds which have an immunomodulatory and/or adjuvant effect.

Such mimetics can be administered alone or in combination with other therapeutics for the treatment of diseases of the present invention.

SCREENS

The substance of the present invention to be used for identifying immunomodulators, adjuvants, mimetics and/or inhibitors of toxin-induced diarrhoea in any of a variety of drug screening techniques. The substance employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The abolition of substance activity or the formation of binding complexes between the substance and the agent being tested may be measured.

Another technique for screening provides for high throughput screening (HTS) of agents having suitable binding affinity to the substances and is based upon the method described in detail in WO 84/03564.

It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

PHARMACEUTICAL COMPOSITIONS

The present invention also provides a pharmaceutical composition comprising administering a therapeutically effective amount of the substance of the present invention and a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof).

The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.

Where the agent is to be delivered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit though the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

VACCINES

In one embodiment of the present invention, the substance is an adjuvant which is incorporated into a vaccine composition used to treat or prevent autoimmune disease, human T cell leukaemia, transplant rejection or graft-versus-host disease (GVHD), allergic or infectious disease.

In another embodiment of the invention, the vaccine composition may additionally comprise an antigen(s) or antigenic determinant(s). Suitable such antigens and/or antigenic determinants are disclosed in WO 99/34817.

Preferably the vaccine composition comprises an antigen and/or antigenic determinant.

Preferably the antigen is a self-antigen or a homologue thereof.

Preferably, the one or more substances of the present invention is used in the preparation of a therapeutic or prophylactic vaccine.

A "prophylactic vaccine" is a vaccine which is administered to naive individuals to prevent disease development.

A "therapeutic vaccine" is a vaccine which is administered to individuals with an existing infection to reduce or minimise the infection or to abrogate the immunopathological consequences of the disease.

The preparation of vaccines which contain one or more substances as an active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents and pH buffering agents.

The vaccine composition may also comprise a combination of adjuvants which enhance the effectiveness of the vaccine. Examples of additional adjuvants which , in combination, may be effective include but are not limited to: aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin,

vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminum hydroxide is approved for human use.

ADMINISTRATION

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient. The dosages below are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited.

The compositions of the present invention may be administered by direct injection. The composition may be formulated for parenteral, mucosal, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each protein may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

The term "administered" includes delivery by viral or non-viral techniques. Viral delivery mechanisms include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectos, herpes viral vectors, retroviral vectors, lentiviral vectors, and baculoviral vectors. Non-viral delivery mechanisms include lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof. The routes for such delivery mechanisms include but are not limited to mucosal, nasal, oral, parenteral, gastrointestinal, topical, or sublingual routes.

The term "administered" includes but is not limited to delivery by a mucosal route, for example, as a nasal spray or aerosol for inhalation or as an ingestable solution; a parenteral route where delivery is by an injectable form, such as, for example, an intravenous, intramuscular or subcutaneous route.

The term "co-administered" means that the site and time of administration of each of the substance of the present invention and an additional entity such as an antigen and/or antigenic determinants are such that the necessary modulation of the immune system is achieved. Thus, whilst the substance and the antigen may be administered at the same moment in time and at the same site, there may be advantages in administering the substance at a different time and to a different site from the antigen. The substance and antigen may even be delivered in the same delivery vehicle - and the substance and the antigen may be coupled and/or uncoupled and/or genetically coupled and/or uncoupled.

The antigenic determinant and peptide or homologue or mimetic thereof may be administered separately or co-administered to the host subject as a single dose or in multiple doses.

The vaccine composition of the invention may be administered by a number of different routes such as injection (which includes parenteral, subcutaneous and intramuscular injection) intranasal, mucosal, oral, intra-vaginal, urethral or ocular administration.

The vaccines comprising the substance of the present invention are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, may be 1% to 2%. Oral formulations include such normally employed excipients as, for example,

pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer

DISEASES

The substance of the present invention is used to treat or prevent autoimmune disease, human T cell leukaemia, transplant rejection, allogeneic or xenogeneic transplant, graft-versus-host disease (GVHD), allergic or infectious diseases. Within the group "infectious diseases", are diseases in which, during infection, the infectious agent binds to, colonises or gains access across the mucosa are particularly preferred, as are diseases in which immunopathological mechanisms are commonly involved.

~~Examples of infectious diseases of the present invention include but are not limited to~~ HSV-1, HSV-2, EBV, VZV, CMV, HHV-6, HHV-7 and HHV-8, hepatitis A, B, C, D and E, Neisseria meningitides, Haemophilus influenzae type B and Streptococcus pneumoniae, Legionella pneumophila and Mycobacterium tuberculosis, Neisseria gonnorheae, HIV-1, HIV-2 and Chlamydia trachomatism, E.coli, rotavirus, Salmonella enteritidis, Salmonella typhi, Helicobacter pylori, Bacillus cereus, Campylobacter jejuni and Vibrio cholerae, Staphylococcus aureus, Streptococcus pyogenes and Streptococcus mutans, malaria, Trypanasoma spp., Taxoplasma gondii, ~~Leishmania donovani and Oncocerca spp.~~

Examples of allergic disorders of the present invention include but are not limited to diseases include asthma, allergic cough, allergic rhinitis and conjunctivitis, atopic eczema and dermatitis, urticaria, hives, insect bite allergy, dietary and certain drug allergies.

Examples of autoimmune diseases include but are not limited to diseases such as rheumatoid arthritis, multiple sclerosis and diabetes.

KITS

The present invention further provides diagnostic assays and kits comprising the substance of the present invention. Such kits may be used to prevent and/or treat and/or modulate the diseases of the present invention.

In one embodiment of the present invention, the kit may also comprise an antigen and/or antigenic determinant and/or a separate adjuvant for coadministration with said therapeutic or prophylactic composition.

Alternatively, a kit may be provided comprising mimetics of the present invention in the form of antibodies of the invention bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

SUMMARY

In summary, the present invention relates to a substance comprising any one or more of: an amino acid sequence comprising the sequence presented as SEQ ID No. 2, or a variant thereof, or a homologue thereof, or a fragment thereof, or a derivative thereof, or a mimetic thereof; which substance is capable of acting in a manner that is the same as or is similar to EtxB and/or CtxB; but wherein the substance does not exhibit GM-1 binding activity.

The present invention also relates to an assay method for determing one or agents that are capable of interacting with and/or affecting the substance of the present invention wherein the assay comprises contacting the substance with an agent to be tested, and then determining whether or not the agent affects the substance.

Other aspects of the present invention are presented below by way of numbered paragraphs which include:

1. A peptide which comprises the sequence EVPGSQH (SEQ ID NO 2), or a homologue or mimetic thereof.

2. A peptide according to paragraph 1, which comprises the sequence VEVPGSQHIDSQ (SEQ ID NO 3).

3. A peptide according to paragraph 2 which comprises the sequence GATFQVEVPGSQHIDSQKKAI (SEQ ID NO 4) or the sequence GETFQVEVPGSQHIDSQKKAI (SEQ ID NO 5).

4. A prophylactic or therapeutic composition which comprises a peptide according to any preceding paragraph or a homologue or mimetic thereof.

5. A prophylactic or therapeutic composition according to paragraph 4, which also comprises an antigen or an antigenic determinant.

6. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used as an adjuvant or immunomodulator.

7. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used to upregulate the production of antibodies at mucosal surfaces.

8. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used to prolong antigen presentation and give sustained immunological memory in a mammalian subject.

9. A prophylactic or therapeutic composition according to paragraph 4 or 5, wherein the therapeutic or prophylactic agent is used to downregulate the pathological components of Th1 and Th2-associated immune responses.

10. A prophylactic or therapeutic composition according to any of paragraph 4 to 9, which is used to treat or prevent autoimmune disease, human T cell leukaemia, transplant rejection, graft-versus-host disease or infectious diseases.

11. A prophylactic or therapeutic composition which comprises an agent which binds specifically to the ß4-α2 loop of EtxB or CtxB.

12. A prophylactic or therapeutic composition according to paragraph 11, wherein the agent is an antibody.

13. A prophylactic or therapeutic composition according to paragraph 11 or 12, which is used to treat diarrhoea.

14. A vaccine composition for use against a disease, comprising a peptide according to any of paragraphs 1 to 3 or a homologue or a mimetic thereof.

15. A vaccine composition according to paragraph 14 which also comprises an antigenic determinant.

16. A vaccine composition according to paragraph 14 or 15 which is used to treat or prevent infections diseases, autoimmune disease, human T cell leukaemia, transplant rejection or graft-versus-host disease (GVHD) diseases.

17. A kit comprising a therapeutic or prophylactic composition according to any of paragraphs 4 to 13.

The present invention will now be described only by way of example in which reference is made to the following Figures:

Figure 1 which shows a stereo ribbon drawing showing a B subunit of Etx/Ctx (from Sixma et al. J. Mol. Biol. (1993) 230:890-918, labels added);

Figure 2 which shows the identification of loop residues in CtxB involved in CD8+ T-cells apoptosis;

Figure 3 which shows mutant B subunits defective in CD8+ T-cell apoptosis retain ability to bind to cell surface receptors;

Figure 4 which shows total immunoglobulin levels against EtxB and EtxB (H57S) in sera from mice immunised intranasally with 10ug of each B-subunit;

Figure 5 which shows that His-57 in CtxB and EtxB defines a region necessary for adjuvanticity;

Figure 6 which shows E51-158 B subunit peptide exhibits an ability to induce immunomodulation of CD8+ T-cells.

EXAMPLES

Example 1

Identification of residues in the Glu-51 to Ile-58 loop of CtxB that trigger immunomodulatory effects on leukocytes

NIH male mice were sacrificed and mesenteric lymph node tissue was subsequently removed into Hanks balanced salt solution (HBSS without Calcium and Magnesium and supplemented with 20mM Hepes). Lymphocytes were then dispersed into the solution and away from fibrous tissue by gently pressing the tissue through a wire mesh. Following 3 washes in HBSS, the lymph node cells were resuspended in modified Eagle's medium (Gibco) (α-MEM) containing 20 mM Hepes, 4mM L-Glutamine, 100 IU/ml penicillin, 100ug/ml streptomycin, and $5x10^{-5}$ M2-

mercaptoethanol, at a concentration 2 x 10^6 cells/ml and then mixed without (PBS control) or with 3.45uM (40 ug/ml) of wild-type CtxB or wild-type EtxB, or various mutant B-subunits, namely EtxB(G33D), CtxB(E51A), CtxB(V52A), CtxB(P53A), CtxB(G54A), CtxB(S55A), CtxB(Q56A), CtxB(H57A), or CtxB(I58A) and incubated at 37°C for 96h. Thereafter, the cells were washed and resuspended in 0.4ml HBSS/20mM Hepes/0.1% sodium azide/10% rat serum. Phycoerythrin (PE) conjugated anti-CD8 (PharMingen) and FITC-conjugated anti-CD4 (PharMingen) were added at a dilution of 1/400 and the cells incubated on ice for a period of 30 min. Following antibody incubations, the cell suspensions were washed once in ISOTON (Becton-Dickinson) and resuspended in 0.4ml ISOTON. FACS analysis was carried out, with 10,000 events collected for each sample and then plotted using WinMDI software. The cells with FITC-bound anti-CD4 are depicted in the top-left hand quadrant of each figure; the cells with PE-bound anti-CD8 are depicted in the bottom right-hand quadrant, with the percentage number of events in each quadrant shown.

Results 1

The results in Figure 2 demonstrate, that incubation of MLN cells with wild-type CtxB or wild-type EtxB causes depletion of CD8+ T-cells; which does not occur if the cells are incubated in the presence of PBS (control) or a mutant form of EtxB, EtxB(G33D) which lacks an ability to bind to cell surface GM-1 ganglioside. An analysis of the CtxB mutants containing Ala substitutions in residues E51 to I58 revealed that CtxB(E51A) and CtxB(H57A) also failed to trigger CD8+ T-cell depletion. In addition, CtxB(V52A) and CtxB(I58A) exhibited a partial defect in triggering CD8+ T-cell depletion. These findings indicate that residues, E51 and H57 play an essential role in triggering modulatory effects on lymphocytes with a contributory role for residues V52 and I58.

Example 2

Mutant B-subunits defective in CD8+ T-cell apoptosis retain ability to bind to cell surface receptors

NIH male mice were sacrificed and mesenteric lymph node tissue was subsequently removed into Hanks balanced salt solution (HBSS without Calcium and Magnesium and supplemented with 20mM Hepes). Lymphocytes were then dispersed into the solution and away from fibrous tissue by gently pressing the tissue through a wire mesh. Following 3 washes in HBSS, the lymph cells were resuspended in 300 ml of pre-cooled and de-gassed MACS buffer (PBS, 5mM EDTA, 0.5% BSA, pH7.2). 50ml each of anti CD4 and anti-B220 MACS antibodies were added to the cells and the CD8 T-cell population purified by negative selection in a magnetic MACS column. The CD8+ T-cells containing population were washed and resuspended in modified Eagle's medium (Gibco) (α-MEM) containing 20 mM Hepes, 4mM L-Glutamine, 100 IU/ml penicillin, 100ug/ml streptomycin, and $5x10^{-5}$ M2-mercaptoethanol, at a concentration 2 x 10^6 cells/ml and then mixed without (PBS control) or with 3.45uM (40 ug/ml) of wild-type CtxB or wild-type EtxB, or various mutant B-subunits, namely EtxB(G33D), CtxB(E51A), CtxB(V52A), CtxB(P53A), CtxB(G54A), CtxB(S55A), CtxB(Q56A), CtxB(H57A), EtxB(H57S), or CtxB(I58A) and incubated on ice for 20 min. Thereafter, the cells were washed and resuspended in ice-cold 0.4ml HBSS/20mM Hepes/0.1% sodium azide/10% rat serum. Anti-EtxB monoclonal antibody 118-8 was added at a dilution of 1/500 to cells incubated EtxB, EtxB(G33D) or EtxB(H57S) and anti-CtxB monoclonal antibody LT-39 was added at a dilution of 1/800 to cells incubated with CtxB and CtxB mutants. After 30 min, the cells were washed and resuspended in HBSS/20mM Hepes/0.1% sodium azide/10% rat serum followed by addition of a FITC-labelled anti-mouse IgG antibody. Following incubation with the secondary antibody for 30 min, the cell suspensions were washed once in ISOTON (Becton-Dickinson) and resuspended in 0.4ml ISOTON. Levels of FITC fluorescence as a representative of the extent of binding of EtxB, CtxB and the various mutants to CD8+ T-cells was assessed by FACS analysis. The results of 10,000 events from each sample are plotted showing the fluorescence

intensity of CD8+ T-cells incubated in the absence of B-subunits (ie PBS contol; red line) versus the fluorescence attributable to bound B-subunits (black line) (Figure 3).

Results 2

The results in Figure 3 show that all B-subunits, except the non-binding mutant EtxB(G33D) bound to CD8+ T-cells to a similar extent. The fluorescence intensity detected after binding CtxB(H57A) and EtxB(H57S) was somewhat higher than that exhibited by wild-type B-subunits indicating that the two mutants have a greater avidity for the cell surface. This result is consistent with the finding that both CtxB(H57A) and EtxB(H57S) bind with a slightly higher avidity to GM-1-coated microtitre plates and exhibit a slightly higher Kd for GM-1 as determined by plasmon surface resonance (data not shown).

Example 3

Residue His-57 in EtxB is required to induce a potent anti-EtxB response

Groups of NIH female mice (n = 8) were immunised intranasally with 10ug EtxB or EtxB(H57S) in a volume of 20ul on 3 occasions at one week intervals. The mice were sacrificed and blood removed by cardiac puncture 14 days after the third immunisation. The sera were analysed for levels of anti-EtxB IgG antibodies by a GM-1-ELISA using microtitre plates coated with 1ug/ml EtxB. End point titres were determined (equivalent to a dilution giving an absorbance of 0.1 above background).

Results 3

The results in Figure 4 show that following intranasal immunisation with wild-type EtxB high titre serum anti-EtxB IgG antibody levels are induced (titre = 5757+/-785) whereas immunisation with EtxB(H57S) induces a significantly (p=0.001) lower response (titre 1205+/-222).

Example 4

Residue His-57 in EtxB and CtxB is necessary for the B-subunits to act as mucosal adjuvants

Groups of NIH female mice (n = 8) were immunised intranasally with 10ug ovalbumin (Ova) alone or with 10ug Ova mixed with either 10ug EtxB, CtxB, EtxB(H57S) or CtxB(H57A) and administered in a volume of 20ul on 3 occasions at one week intervals. In addition, two groups of mice were intranasally immunised with 10ug EtxB or CtxB as negative controls. All mice were sacrificed 14 days after the third immunisation and the blood removed by cardiac puncture. The sera were then analysed for levels of anti-Ova IgG antibodies by an ELISA using microtitre plates coated with 5ug/ml Ova. End point titres were determined (equivalent to a dilution giving an absorbance of 0.1 above background).

Results 4

The results in Figure 5 show that both wild-type EtxB and CtxB act as mucosal adjuvants, substantially augmenting the anti-Ova response compared with mice immunised with Ova alone (compare lanes 4 and 5 with lane 1). By contrast, when Ova was admixed with EtxB(H57S) (lane 6) or CtxB(H57A) (lane 7) the anti-Ova response induced was substantially less than that triggered by inclusion of the wild-type B-subunits. The data demonstrate that CtxB(H57A) lacks adjuvant activity. This confirms the importance of the B-subunit E51-I58 loop, and in particular H57 in mediating the immunomodulatory properties of the molecule.

Example 5

A synthetic peptide EVPGSQHI corresponding to the E51 to I58 loop of EtxB and CtxB possesses immunomodulatory properties

To assess whether a synthetic peptide corresponding to the E51 to I58 loop of EtxB (and CtxB) causes depletion of CD8+ T-cells from MLN cultures, mesenteric lymph node cells were isolated as described in Example 1, and incubated with various concentrations (0.1uM - 20uM) of the EVPGSQHI (SEQ ID NO 2 with a carboxyl - terminal isoleucine) or a randomly selected control peptide, LRNETTTTKGDYC (SEQ ID NO 6). After 96h incubation at 37°C the cells were washed and resuspended in 0.4ml HBSS/20mM Hepes/0.1% sodium azide/10% rat serum and then assessed for the relative proportion of CD4+ and CD8+ cells by FACS analysis, in an identical fashion to that reported in Example 1. The percentages of CD8+ T-cells remaining in cultures treated with the EVPGSQHI peptide (closed red circles and line) or the control peptide (closed black squares and line) was determined and was plotted graphically against concentration of peptide used (Figure 6).

Results 5

The results in Figure 6 show that incubation of MLN cultures in the presence of the EVPGSQHI peptide causes a reduction in CD8+ T-cell numbers, in contrast to treatment with a control peptide. This shows that a synthetic peptide corresponding to the E51 to I58 loop of EtxB and CtxB is active in exerting direct modulatory effects on lymphocytes.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.